Original Article

Comparative sensitivity analysis between two methods for species differentiation and interspecies cross contamination in animal cell culture

Ashraf Mohammadi[1*], Mansooreh Farzinpoor[2], Mohsen Lotfi[3], Raiehaneh Ghorbani[4]

[1] Razi Vaccine and Serum Research Institute (RVSRI) Hessark Karadj Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran.
[2] Razi Vaccine and Serum Research Institute (RVSRI) Hessark Karadj Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran.
[3] Razi Vaccine and Serum Research Institute (RVSRI) Hessark Karadj Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran.
[4] Razi Vaccine and Serum Research Institute (RVSRI) Hessark Karadj Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran.

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Abstract

Background: The present article focuses on the design and development of a highly sensitive and convenient approach for rapid detection of animal species and cross contaminations quickly during cell cultures as most important document for manufacturing working cell bank system. This test is one of the four most important documents during implementing the banking system. By using this modified test, one of the major risks in cell culture laboratories, cross- contamination and misidentifications with microorganisms of cell lines will also be important to be confirmed.

Materials and Methods: A PCR _RFLP assay was optimized based on the use of a pair of primers that anneal to a portion the cytochrome b gene in all the species. The amplification product was digested with a panel of six restriction enzymes and the pattern derived was resolved on 3% high resolution agarose gel for 2 species, human & primate. As a control test iso enzyme assay as a conventional method was used.

Results: This protocol produced a unique restriction pattern and the origin was confirmed by this analysis. The sensitivity in detecting interspecies cross contamination was at least 100 pg DNA/reaction, which was sufficient for detection of each species of DNA.

Conclusion: The method developed in this study will provide a useful tool for the authentication of animal species and is also more comparable and time consuming, compared with conventional analysis. Using this method, significant differences between human and non-human as well as cross- contamination between different cell lines are simply distinguished.

Keywords: cell authentication cross contamination, PCR-RFLP assay, restriction enzyme

***Corresponding Author:** Ashraf Mohammadi ;DVM,PhD; Associate Professor- Razi Vaccine and Serum Research Institute (RVSRI) Hessark Karadj Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran:Tel(+98)2134570038-233. Fax(+98) 21 34572194; Email: A.mohammadi@rvsri.ac.ir

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Introduction

Cell lines have wide applications as model system in the medical and pharmaceutical industry.

Each biological product such as vaccine or

antigens (1) involving the use of cell lines, require complete knowledge of the species origin. Each cell line has unique features and can be used for specific studies. It is therefore important to know characteristics of the cell lines and link the description of their availability. Comprehensive testing regimens for detection of adventitious agents in vaccine cell substrates are designed to minimize the risk of viruses and cell to cell cross contamination in vaccines that assure product safety. There are some methods used for this purpose, alloenzyme and isoenzyme analysis (2, 3), Karyotyping analysis (4), human leukocyte antigen typing (5) immune cytochemical analysis (6)DNA fingerprinting (7-10).These tests not only require well-trained and experienced personnel but also contain high variability low reproducibility and also are expensive and time consuming. A polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assay for rapid identification and authentication of different cell line was optimized. In this method, a pair of primers was used to anneal to a portion of cytochrome b gene for differentiation between 6 different species, human, rabbit, dog, monkey and chicken embryo fibroblast. The result of this assay in compare of designing specific primer for each species (11) provides a rapid, simple, sensitive, and cost-effective method for species differentiation and interspecies cross contamination in cell culture.

Methods

Cell lines. Continuous cell lines analyzed in this work are collected in RAZI human viral vaccine cell bank system. Cell lines/tissue tested for each species stored in liquid nitrogen, considering to the test were thawed at 37°C diluted in DMEM contain 5-7% calf serum and centrifuged 180×g; the cell pellets re suspend in 200 µl of phosphate – buffered slain. All samples analyzed are listed in (Table1). Primary Chicken embryo fibroblast cultures were prepared based on protocol from specific pathogen free 9-11 day eggs.

DNA extraction. DNA extraction from cell culture, and tissues was performed with Yekta Tajhiz azma, DNA extraction kit, according to manufacturer's protocol. (Fig-1)PCR-RFLP: Primers Ra4816 and Rb5173 were designed for amplification of 358 bp length fragment of cytochrome b gene of all species. Primers sequences are

-Ra4816F5´CCATCCTGCAAACATCTCTCA TGAAA -3´ and Rb15173R5´-CCCCTCCATAAGAATTTTCGC CTCA-3´. The amplification was performed in a final volume of 50µl; PCR condition were: MgCl2, 2 mM,deoxyribonucleotide triphosphates(dNTPs) 0.2 mM each, forward primer 0.5 mM, reverse primer 0.5 mM, Taq Gold 3U. The PCR protocol was: 95°C for 12 min followed by 30 cycles of 95°C for 40 s, 44°C for 40 s, 72°C for 40 s, and final extension at72° C for 5 min.

RFLP analysis of PCR product. Ten micro liters of PCR products were digested with 10 U of AluI, HinfI, HaeIII, TaqI, RsaI and MboI in a final volume of 29 µl for 3 h at 37°C for all the endonucleases with the exception of TagI, which required a temperature of 65°C (Figure 2).

PCR-RFLP sensitivity. To determine sensitivity of test in detecting interspecies cross contaminations, DNA from two different species, human and monkey were mixed in different proportions, maintaining the total amount of DNA equal to 100 ng per reactions.) In this case, contaminant (monkey) DNA proportions were: 100–90–80-70–5030–20–10–5–2.5%, to the following DNA amounts of Human: 2.5, 10, 20, 30, 50, 70, 80, 90, and 97.5 ng. (Figure 3)

Species-specific PCR. Using the Coopers method (11) the species specific primers were prepared to amplify specific sequences for human, dog, CHEF, Rabbit and Rhesus monkey. (Figure 4)

Gel Electrophoresis. The fragments resulting from the digestion were resolved on a 3% high-resolution agarose gel (Roche, Germany), with 0.05 µg/ml ethidium bromide in 1× Tris-acetate-ethylenediamine tetraacetic acid (TAE) buffer for 60 min at 180V and visualized with a UV transilluminator.

Results

The primers were targeted a portion of cytochrome b gene and allowed the amplification of a

Table1: cell lines/tissue tested for each species (ECL: Established cell line)

Human	MRC-5	Lung	ECL
Human	FSC-2	Foreskin	ECL
Monkey(Rhesus)	RMK-2	Kidney	ECL
Monkey(Rhesus)	VERO	Kidney	ECL
Rabbit	RK-13	Kidney	ECL
Dog	MDCK	Kidney	ECL

358 bp fragment in all six different cell lines above mentioned. PCR product electrophoresis did not show any additional band.

The sensitivity of the PCR reaction, was evaluated for each species analyzed, using as two series of templates regarding different DNA dilutions were estimated at 2−10 ng DNA per reaction in all the species. The restriction enzyme chosen were as following: AluI, HinfI, HaeIII, TaqI, RsaI and Mob; table 2 describes the expected restriction pattern of 6 different species. Electrophoresis profile of PCR-RFLP on DNA from different species showed differentiated pattern for different cell lines completely (Figure 2). In this work, the ability of PCR-RFLP to identify human from monkey species as well as detecting interspecies cross contamination were evaluated (Figure 3). The sensitivity of the PCR-RFLP reaction, was evaluated using the couple of primers targeted to a portion of the specific sequence for each species. These primers were allowed the amplification of specific fragment, for each Species (Figure 4).

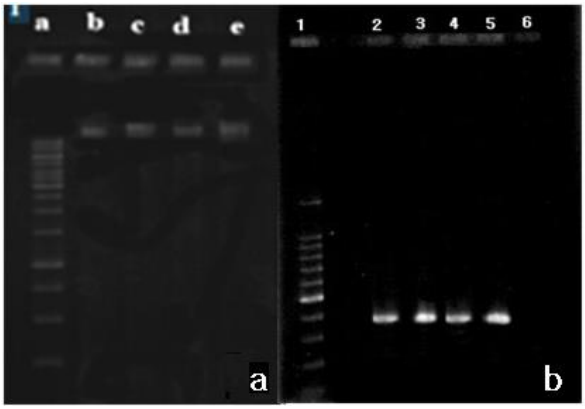

Figure 1. a) Electrophoresis profile in 1.5% agarose gel of DNA extract.b Electrophoresis profile in 1.5% agarose gel of PCR on a 358 bp length fragment of the cytochrome b gene.(Lane 1a:1kbp ladder, lane 2a: 100bp ladder, lane b: MRC-5, lane c: fsc-2, lane d: RMK-2, lane e: VERO, lane f: negative control PCR

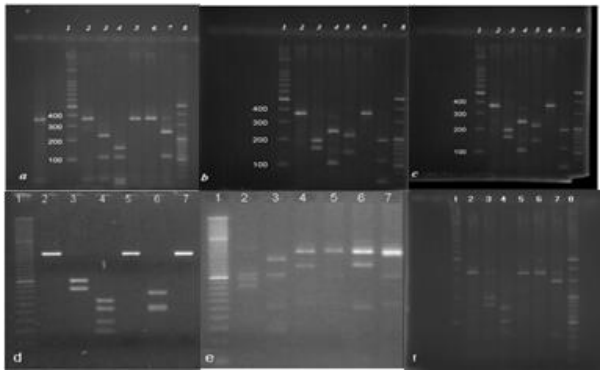

Figure 2. Electrophoresis profile on 3% low melts grose gel of PCR-RFLP on DNA fragment of cytochrome b gene. a. RK-13 cell line, b.MRC-5 cell line, c.FSC-2 cell line d.CHEF culture e. MDCK cell Line, f. VERO cell line.(lane 1: 100bp ladder, lane b: AluI, lane c: HinfI, lane d: HaeIII, lane e: TaqI, lane f: RsaI, lane g: MboI, lane 8: 20bp ladder)

Discussion

We have developed a simple, sensitive, and rapid PCR-RFLP assay that can be used to efficiently identify common cell cultures.

The described assay can greatly reduce the risk of most interspecies contamination in a PCR-RFLP reaction and is therefore recommended for labs frequently using cell lines in their research.

By employing these assays, species identification of cell cultures becomes a rapid, cost-effective routine procedure, and it overcomes the limitations of the currently used methodology. Many published articles on cell biology may be based on cell lines that have not been properly validated. In one estimate, 18-36 percent of all cell lines are contaminated, and the International Cell Line Authentication Committee currently lists 475 cross-contaminated or misidentified cell lines in its database. Error-ridden papers are frequently published and cited, funding and resources can be wasted on research projects developed with inaccurate findings (11-14)

Based on only two contaminated cell lines (HEp-2 and INT 407), it has been estimated that approximately $713M has been spent on work that produced 7,125 publications using these erroneous cell lines. Methods used for cell line authentication are (a) the traditional method of iso enzyme analysis, which detects interspecies contamination of at least 10 % (7);

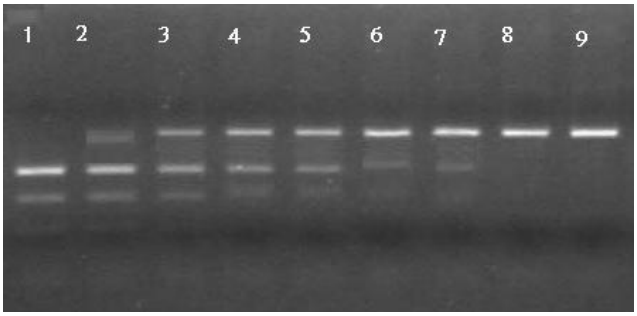

Figure 3. A Electrophoretic profile on 3% low-melt agarose gel of PCRRFLPReactions performed using DNA mixtures of human (Human Lung Diploid Cell Strain) MRC-5and monkey cell lines (Razi Monkey Continuous Cell Line) RMKas a template for PCR. PCR products are digested only with MboI 100% lane 1: Human DNA, lane 2: 90% Human DNA+10% monkey DNA, lane 3: 50%Human DNA+50% monkey DNA, lane 4: 33% Human DNA+67% monkey DNA, lane 5: 25% Human DNA+75% monkey DNA, lane 6: 10% Human DNA+90%monkey DNA, lane 7: 5% Human DNA+95% monkey DNA, lane 8: 2.5% HumanDNA+97.5% monkey DNA, lane 9: 100% monkey DNA

(b) DNA fingerprinting based on the specific pattern of polymorphisms of individual DNA samples (exclusion rates of 99% or higher) (8)

(c) Cytogenetic analysis (karyotyping), which is currently considered the most sensitive method for the identification of interspecies contamination. However, it is a labor-intensive, time-consuming and a rather expensive procedure (8). Manufacturing stages which should be tested for cell to cell cross contamination including the testing of master cell bank, limited testing of the working cell bank and end of the production of biological products.

In the 1950s and 1960s, many continuous cell lines were unknowingly cross-contaminated with other cell lines including HeLa cells. In 1970s and 1980s as many as one in three cell lines deposited in cell banks were contaminated.

This cross-contamination was only uncovered with the development of suitable genetic markers beginning in 1967s. More recently, cell repositions have used DNA polymorphisms in addition to enzyme polymorphism, HLA typing and karyotyping to confirm the identity of their cell lines. In our previous publication, polymerase chain reaction (PCR) has been used for identification of a new cell line, including methods for cell line authentication (15). In this study, an alternative method to

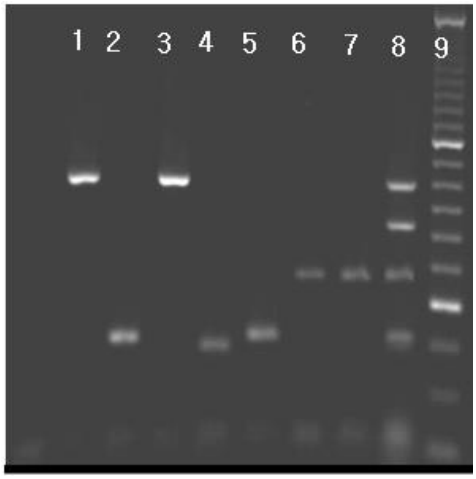

Figure 4. Detection of species-specific amplified products in a specific PCR assay (cooper 2007). Lane 1. MRC-5, Lane2. Rabbit, Lane 3. FSC-2 Lane 4. Rabbit, Lane 5.MDCK. Lane6.RMK Lane 7. Lab control lane 8. 20bp ladder 9. 100bp ladder

isoenzyme analysis was developed based on PCR-RFLP. This technique requires the amplification of a portion (358bp) of cytochrome b gene and permits the authentication of cell line belonging to primate and human. A panel of 6 cell lines was tested (Table 1) whose species of origin had been previously confirmed by isoenzyme biochemical analysis. In all the experiments, the cell lines, when tested with the species specific primer) pair (11), showed a band of amplification corresponding to the expected size of the fragment amplified, while no amplification was seen when primer pairs specific for different species were used. As biological sample DNA from cell line was used as template for PCR-RFLP assay. The amplification product was digested with a panel of six restriction enzyme and the pattern derived was resolved on 3% high resolution agarose gel.

The result of the current study suggest that cytochrome b gene PCR-RFLP is a less expensive and time consuming alternative to isoenzyme analysis. This protocol produced a unique restriction pattern and the origin of this animal cells resulted to be confirmed by this analysis. Its sensitivity in detecting interspecies cross contamination was at least100 pg DNA/reaction, which was sufficient for detection of each species of DNA. The method developed in this study will provide a useful tool for authentication of animal species which is more comparable, more time consuming and having the high cost benefit compared

with other analysis.

At present, this technique is performed for identification of different cell line from different origin in parallel with other assay in RAZI human viral vaccine laboratory for characterization the manufacturing cell bank system.

Conflicts of Interest

There is no conflict of interest.

Acknowledgment

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